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(54) Title: DESIGN OF HIGH AFFINITY RNASE H RECRUITING OLIGONUCLEOTIDE

(57) Abstract: The present invention relates to the field of bicyclic DNA analogues, e.g. LNA and LNA modifications, which are useful for designing oligomers that form high affinity duplexes with complementary RNA wherein said duplexes are substrates for RNase H. The oligonucleotides may be partially or fully composed of LNA analogues with very high affinity and ability to recruit RNase H. The implications are that oxy-LNA by itself may be used to construct novel antisense molecules with enhanced biological activity. Alternatively, oxy-LNA may be used in combination with non-oxy-LNA, such as standard DNA, RNA or other analogues, e.g. thio-LNA or amino-LNA, to create high affinity, RNase H recruiting anti-sense compounds without the need to adhere to any fixed design.

Design of high affinity RNase H recruiting oligonucleotide

Field of Invention

The present invention relates to the field of bicyclic DNA analogues which are useful for designing oligomers that forms high affinity duplexes with complementary RNA wherein said duplexes are substrates for RNase H. The oligonucleotides may be partially or fully composed of bicyclic DNA analogues.

Background of the invention

The term "antisense" relates to the use of oligonucleotides as therapeutic agents. Briefly, an antisense drug operates by binding to the mRNA thereby blocking or modulating its translation into protein. Thus, antisense drugs may be used to directly block the synthesis of disease causing proteins. It may, of course, equally well be used to block synthesis of normal proteins in cases where these participate in, and aggravate a pathophysiological process. Also, it ought to be emphasised that antisense drugs can be used to activate genes rather that suppressing them. As an example, this can be achieved by blocking the synthesis of a natural suppressor protein.

Mechanistically, the hybridising oligonucleotide is thought to elicit its effect by either creating a physical block to the translation process or by recruiting a cellular enzyme (RNase H) that specifically degrades the mRNA part of the mRNA/antisense oligonucleotide duplex.

Not unexpectedly, oligonucleotides must satisfy a large number of different requirements to be useful as antisense drugs. Importantly, the antisense oligonucleotide must bind with high affinity and specificity to its target mRNA, must have the ability to recruit RNase H, must be able to reach its site of action within the cell, must be stable to extra – and intracellular nucleases both endo- and exo-nucleases, must be non-toxic/minimally immune stimulatory, etc.

Natural DNA only exhibit modest affinity for RNA and fall short on a number of the other critical characteristics, especially nuclease resistance. Hence, a significant effort has been invested to identify novel analogues with improved antisense properties. In particular the search has focused on identifying novel analogues, which combine an increased affinity

for complementary nucleic acids with the RNase H recruiting ability of natural DNA. Both of these properties have been demonstrated to correlate in a strongly positive manner with biological activity. Of the vast number of analogues that have emerged from this work, only few retain the ability to recruit RNase H and very few provide useful increases in affinity. Sadly, those that do provide a useful increase in affinity fail to recruit RNase H.

In the face of these results the field have turned to mixed backbone oligonucleotides as a means to provide higher potency antisense drugs, *i.e.* antisense molecules that operates by a two fold mechanism of action 1) high affinity mediated translational arrest at the ribosomal level and 2) activation of RNase H. These molecules (termed gab-mers) typically comprise a central region of at least six contiguous, low affinity phosphorothioates (RNase H recruiting analogues) flanked by stretches of high affinity analogues (non RNase H recruiting analogues) that enhance the ability to promote translational arrest. Although expected to out-perform current phosphorothioate antisense drugs, the gab-mers are not considered the ideal antisense molecules. Amongst their weaknesses is the requirement for a rather fixed design and the presence of high and low affinity domains within the molecule, which may compromise biological activity.

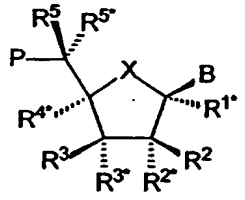
The enzyme RNase H selectively binds to heterogeneous DNA/RNA duplexes and degrades the RNA part of the duplex. Homogeneous DNA/DNA and RNA/RNA duplexes, which only differs molecularly from the DNA/RNA duplex at the 2′ position (**DNA/DNA:** 2′-H/2′-H; **RNA/RNA:** 2′-OH/2′-OH and **DNA/RNA:** 2′-H/2′-OH) are not substrates for the enzyme. This suggests that either the molecular composition at the 2′ position itself or the structural feature it imposes on the helix is vital for enzyme recognition. Consistent with this notion, all 2′-modified analogues that have so far been reported to exhibit increased affinity have lost the ability to recruit RNase H.

Detailed description of the invention.

Locked Nucleic Acid (LNA) is a novel, nucleic bicyclic acid analogue in which the 2′- and 4′ position of the furanose ring are linked by an O-methylene (oxy-LNA), S-methylene (thio-LNA) or NH_2-methylene moiety (amino-LNA). This linkage restricts the conformational freedom of the furanose ring and leads to an increase in affinity which is by far the highest ever reported for a DNA analogue (WO 99/14226).

Despite the fact that the modification in LNA involves the 2'-position we have found that the activity of RNase H is not dependent on a contiguous stretch of DNA or phosphorothioated bases when oxy-LNA is used as a component of the oligonucleotide. In fact, we have found that oligonucleotides composed entirely of oxy-LNA are able to recruit RNase H. Oxy-LNA oligonucleotides thus constitutes the first ever DNA analogue to display the long sought after combination of very high affinity and ability to recruit RNase H. The implications are that oxy-LNA by itself may be used to construct novel antisense molecules with enhanced biological activity. Alternatively, oxy-LNA may be used in combination with non-oxy-LNA, such as standard DNA, RNA or other analogues, e.g. thio-LNA or amino-LNA to create high affinity, RNase H recruiting antisense compounds without the need to adhere to any fixed design.

An "oxy-LNA monomer" is defined herein as a nucleotide monomer of the formula Ia



Ia

wherein X is oxygen; B is a nucleobase; R^{1*}, R^2, R^3, R^5 and R^{5*} are hydrogen; P designates the radical position for an internucleoside linkage to a succeeding monomer, or a 5'-terminal group, R^{3*} is an internucleoside linkage to a preceding monomer, or a 3'-terminal group; and R^{2*} and R^{4*} together designate -O-CH_2- where the oxygen is attached in the 2'-position.

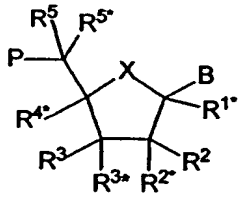
The term "nucleobase" covers the naturally occurring nucleobases adenine (A), guanine (G), cytosine (C), thymine (T) and uracil (U) as well as non-naturally occuring nucleobases such as xanthine, diaminopurine, 8-oxo-N^6-methyladenine, 7-deazaxanthine, 7-deazaguanine, N^4,N^4-ethanocytosin, N^6,N^6-ethano-2,6-diaminopurine, 5-methylcytosine, 5-(C^3-C^6)-alkynylcytosine, 5-fluorouracil, 5-bromouracil, pseudoisocytosine, 2-hydroxy-5-methyl-4-triazolopyridin, isocytosine, isoguanin, inosine and the "non-naturally occurring" nucleobases described in Benner et al., U.S. Pat No. 5,432,272 and Susan M. Freier and Karl-Heinz Altmann, Nucleic Acids Research, 1997, vol. 25, pp 4429-4443. The term "nucleobase" thus includes not only the known purine and pyrimidine heterocycles, but also heterocyclic analogues and tautomers thereof. It should be clear to the person skilled in

the art that various nucleobases which previously have been considered "non-naturally occurring" have subsequently been found in nature.

A "non-oxy-LNA" monomer is broadly defined as any nucleoside (i.e. a glycoside of a heterocyclic base) which is not itself an oxy-LNA but which can be used in combination with oxy-LNA monomers to construct oligos which have the ability to bind sequence specifically to complementary nucleic acids. Examples of non-oxy-LNA monomers include 2'-deoxynucleotides (DNA) or nucleotides (RNA) or any analogues of these monomers which are not oxy-LNA, such as for example the thio-LNA and amino-LNA described by Wengel and coworkers (Singh et al. J. Org. Chem. 1998, 6, 6078-9, and the derivatives described in Susan M. Freier and Karl-Heinz Altmann, Nucleic Acids Research, 1997, vol 25, pp 4429-4443.

It should be understood that the incorporation of non-oxy-LNA monomer(s) into an oxy-LNA oligo may change the RNAseH recruiting characteristics of the oxy-LNA/non-oxy-LNA chimeric oligo. Thus, depending on the number and type of non-oxy-LNA monomer(s) used, and the position of these monomers in the resulting oxy-LNA/non-oxy-LNA chimeric oligo, the chimera may have an increased, unaltered or decreased ability to recruit RNAsdeH as compared to the corresponding all oxy-LNA oligo.

As mentioned above, a wide variety of modifications of the deoxynucleotide skeleton can be contemplated and one large group of possible non-oxy-LNA can be described by the following formula I



I

wherein X is -O-; B is selected from nucleobases; R^{1*} is hydrogen;

P designates the radical position for an internucleoside linkage to a succeeding monomer, or a 5'-terminal group, such internucleoside linkage or 5'-terminal group optionally including the substituent R^5, R^5 being hydrogen or included in an internucleoside linkage,

R^{3*} is a group P* which designates an internucleoside linkage to a preceding monomer, or a 3'-terminal group;

one or two pairs of non-geminal substituents selected from the present substituents of R^2, R^{2*}, R^3, R^{4*}, may designate a biradical consisting of 1-4 groups/atoms selected from $-C(R^aR^b)-$, $-C(R^a)=C(R^a)-$, $-C(R^a)=N-$, $-O-$, $-S-$, $-SO_2-$, $-N(R^a)-$, and $>C=Z$,

wherein Z is selected from -O-, -S-, and $-N(R^a)-$, and R^a and R^b each is independently selected from hydrogen, optionally substituted C_{1-6}-alkyl, optionally substituted C_{2-6}-alkenyl, hydroxy, C_{1-6}-alkoxy, C_{2-6}-alkenyloxy, carboxy, C_{1-6}-alkoxycarbonyl, C_{1-6}-alkylcarbonyl, formyl, amino, mono- and di(C_{1-6}-alkyl)amino, carbamoyl, mono- and di(C_{1-6}-alkyl)-amino-carbonyl, amino-C_{1-6}-alkyl-aminocarbonyl, mono- and di(C_{1-6}-alkyl)amino-C_{1-6}-alkyl-aminocarbonyl, C_{1-6}-alkyl-carbonylamino, carbamido, C_{1-6}-alkanoyloxy, sulphono, C_{1-6}-alkylsulphonyloxy, nitro, azido, sulphanyl, C_{1-6}-alkylthio, halogen, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands,

said possible pair of non-geminal substituents thereby forming a monocyclic entity together with (i) the atoms to which said non-geminal substituents are bound and (ii) any intervening atoms; and

each of the substituents R^2, R^{2*}, R^3, R^{4*} which are present and not involved in the possible biradical is independently selected from hydrogen, optionally substituted C_{1-6}-alkyl, optionally substituted C_{2-6}-alkenyl, hydroxy, C_{1-6}-alkoxy, C_{2-6}-alkenyloxy, carboxy, C_{1-6}-alkoxycarbonyl, C_{1-6}-alkylcarbonyl, formyl, amino, mono- and di(C_{1-6}-alkyl)amino, carbamoyl, mono- and di(C_{1-6}-alkyl)-amino-carbonyl, amino-C_{1-6}-alkyl-aminocarbonyl, mono- and di(C_{1-6}-alkyl)amino-C_{1-6}-alkyl-aminocarbonyl, C_{1-6}-alkyl-carbonylamino, carbamido, C_{1-6}-alkanoyloxy, sulphono, C_{1-6}-alkylsulphonyloxy, nitro, azido, sulphanyl, C_{1-6}-alkylthio, halogen, photochemically active groups, thermochemically active groups, chelating groups, reporter groups, and ligands;

and basic salts and acid addition salts thereof;

with the proviso the monomer is not oxy-LNA.

Particularly preferred non-oxy-LNA monomers are 2'-deoxyribonucleotides, ribonucleotides, and analogues thereof that are modified at the 2'-position in the ribose, such as 2'-

O-methyl, 2'-fluoro, 2'-trifluoromethyl, 2'-O-(2-methoxyethyl), 2'-O-aminopropyl, 2'-O-dimethylamino-oxyethyl, 2'-O-fluoroethyl or 2'-O-propenyl, and analogues wherein the modification involves both the 2'and 3' position, preferably such analogues wherein the modifications links the 2'- and 3'-position in the ribose, such as those described by Wengel and coworkers (Nielsen et al., J. Chem. Soc., Perkin Trans. 1, 1997, 3423-33, and in WO 99/14226), and analogues wherein the modification involves both the 2'and 4' position, preferably such analogues wherein the modifications links the 2'- and 4'-position in the ribose, such as analogues having a $-CH_2-S-$ or a $-CH_2-NH-$ or a $-CH_2-NMe-$ bridge (see Wengel and coworkers in Singh et al. J. Org. Chem. 1998, 6, 6078-9). Although, non-oxy-LNA monomers having the β-D-ribo configuration are often the most applicable, further interesting examples (and in fact also applicable) of non-oxy-LNA are the stereoisomeric of the natural β-D-ribo configuation. Particularly interesting are the α-L-ribo, the β-D-xylo and the α-L-xylo configurations (see Beier et al., Science, 1999, 283, 699 and Eschenmoser, Science, 1999, 284, 2118), in particular those having a 2'-4' $-CH_2-S-$, $-CH_2-NH-$, $-CH_2-O-$ or $-CH_2-NMe-$ bridge (see Wengel and coworkers in Rajwanshi et al., Chem. Commun., 1999, 1395 and Rajwanshi et al., Chem. Commun., 1999, submitted)

In the present context, the term "oligonucleotide" which is the same as "oligomer" which is the same as "oligo" means a successive chain of nucleoside monomers (*i.e.* glycosides of heterocyclic bases) connected via internucleoside linkages. The linkage between two successive monomers in the oligo consist of 2 to 4, preferably 3, groups/atoms selected from $-CH_2-$, -O-, -S-, $-NR^H-$, >C=O, $>C=NR^H$, >C=S, $-Si(R'')_2-$, -SO-, $-S(O)_2-$, $-P(O)_2-$, $-PO(BH_3)-$, -P(O,S)-, $-P(S)_2-$, -PO(R")-, $-PO(OCH_3)-$, and $-PO(NHR^H)-$, where R^H is selected form hydrogen and C_{1-4}-alkyl, and R" is selected from C_{1-6}-alkyl and phenyl. Illustrative examples of such linkages are $-CH_2-CH_2-CH_2-$, $-CH_2-CO-CH_2-$, $-CH_2-CHOH-CH_2-$, $-O-CH_2-O-$, $-O-CH_2-CH_2-$, $-O-CH_2-CH=$ (including R^5 when used as a linkage to a succeeding monomer), $-CH_2-CH_2-O-$, $-NR^H-CH_2-CH_2-$, $-CH_2-CH_2-NR^H-$, $-CH_2-NR^H-CH_2-$, $-O-CH_2-CH_2-NR^H-$, $-NR^H-CO-O-$, $-NR^H-CO-NR^H-$, $-NR^H-CS-NR^H-$, $-NR^H-C(=NR^H)-NR^H-$, $-NR^H-CO-CH_2-NR^H-$, -O-CO-O-, -O-CO-CH_2-O-, $-O-CH_2-CO-O-$, $-CH_2-CO-NR^H-$, -O-CO-NR^H-, $-NR^H-CO-CH_2-$, $-O-CH_2-CO-NR^H-$, $-O-CH_2-CH_2-NR^H-$, -CH=N-O-, $-CH_2-NR^H-O-$, $-CH_2-O-N=$ (including R^5 when used as a linkage to a succeeding monomer), $-CH_2-O-NR^H-$, $-CO-NR^H-CH_2-$, $-CH_2-NR^H-O-$, $-CH_2-NR^H-CO-$, $-O-NR^H-CH_2-$, $-O-NR^H-$, $-O-CH_2-S-$, $-S-CH_2-O-$, $-CH_2-CH_2-S-$, $-O-CH_2-CH_2-S-$, $-S-CH_2-CH=$ (including R^5 when used as a linkage to a succeeding monomer), $-S-CH_2-CH_2-$, $-S-CH_2-CH_2-O-$, $-S-CH_2-CH_2-S-$, $-CH_2-S-CH_2-$, $-CH_2-SO-CH_2-$, $-CH_2-SO_2-CH_2-$, -O-SO-O-, $-O-S(O)_2-O-$, $-O-S(O)_2-CH_2-$, $-O-S(O)_2-$

NR^H-, -NR^H-$S(O)_2$-CH_2-, -O-$S(O)_2$-CH_2-, -O-$P(O)_2$-O-, -O-P(O,S)-O-, -O-$P(S)_2$-O-, -S-$P(O)_2$-O-, -S-P(O,S)-O-, -S-$P(S)_2$-O-, -O-$P(O)_2$-S-, -O-P(O,S)-S-, -O-$P(S)_2$-S-, -S-$P(O)_2$-S-, -S-P(O,S)-S-, -S-$P(S)_2$-S-, -O-PO(R")-O-, -O-$PO(OCH_3)$-O-, -O-PO-(OCH_2CH_3)-O-, -O-$PO(OCH_2CH_2S$-R)-O-, -O-$PO(BH_3)$-O-, -O-$PO(NHR^N)$-O-, -O-$P(O)_2$-NR^H-, -NR^H-$P(O)_2$-O-, -O-$P(O,NR^H)$-O-, -CH_2-$P(O)_2$-O-, -O-$P(O)_2$-CH_2-, and -O-$Si(R")_2$-O-; among which -CH_2-CO-NR^H-, -CH_2-NR^H-O-, -S-CH_2-O-, -O-$P(O)_2$-O-, -O-P(O,S)-O-, -O-$P(S)_2$-O-, -NR^H-$P(O)_2$-O-, -O-$P(O,NR^H)$-O-, -O-PO(R")-O-, -O-$PO(CH_3)$-O-, and -O-$PO(NHR^N)$-O-, where R^H is selected form hydrogen and C_{1-4}-alkyl, and R" is selected from C_{1-6}-alkyl and phenyl, are especially preferred. Further illustrative examples are given in Mesmaeker et. al., Current Opinion in Structural Biology 1995, 5, 343-355 and Susan M. Freier and Karl-Heinz Altmann, Nucleic Acids Research, 1997, vol 25, pp 4429-4443. The left-hand side of the internucleoside linkage is bound to the 5-membered ring as substituent P^* at the 3'-position, whereas the right-hand side is bound to the 5'-position of a preceding monomer.

The term "succeeding monomer" relates to the neighbouring monomer in the 5'-terminal direction and the "preceding monomer" relates to the neighbouring monomer in the 3'-terminal direction.

Monomers are referred to as being "complementary" if they contain nucleobases that can form hydrogen bonds according to Watson-Crick base-pairing rules (e.g. G with C, A with T or A with U) or other hydrogen bonding motifs such as for example diaminopurine with T, inosine with C, pseudoisocytosine with G, etc.

When the modified oxy-LNA oligo contain at least two non-oxy-LNA monomers these may contain the same or different nucleobases at the 1'-position and be identical at all other positions or they may contain the same or different nucleobases at the 1'-position and be non-identical at at least one other position.

Accordingly, the present invention describes a method for degrading RNA *in-vivo* (in a cell or organism) or *in-vitro* by providing a high affinity oligonucleotide which activates RNaseH when the high affinity oligonucleotide is hybridised to a complementary RNA target sequence, said high affinity oligonucleotide may consist of oxy-LNA monomers exclusively.

Alternatively, the high affinity oligonucleotide may also consist of both oxy-LNA and non-oxy-LNA monomers, in this case the high affinity oligonucleotide contains at the most five, e.g. 4, e.g. 3 , e.g. 2 contiguous non-oxy-LNA monomers at any given position in the oligonucleotide, e.g. said high affinity oligonucleotide consists of both oxy-LNA and non-oxy-LNA monomers, wherein none of the non-oxy-LNA monomers are located adjacent to each other.

The high affinity oligonucleotide may also contain one or more segments of contigous non-oxy-LNA monomers. For instance, a stretch of contigous non-oxy-LNA monomers may be located in the centre of the oligonucleotide and with flanking segments consisting of oxy-LNA monomers. Alternatively the stretch of contigous non-oxy-LNA monomers may be located at either or both ends. Also, the oxy-LNA segement(s) may be either contigous or interrupted by 1 or more non-oxy-LNA monomers. Also, the high affinity oligonucleotide may comprise more than one type of internucleoside linkage such as for example mixes of phosphordiester and phosphorothioate linkages.

The resulting high affinity oligo containing oxy-LNA monomers and/or non-oxy-LNA monomers can thus be characterized by the general formula

$$5'\text{-}[X_mY_nX_p]_q\text{-}3'$$

X is oxy-LNA and Y is non-oxy-LNA, wherein m and p are integers from 0 to 30, n is an integer from 0 to 3 and q is an integer from 1 to 10 with the proviso that the sum of m+n+p multiplied with q is in the range of 6-100, such as 8, e.g. 9, e.g. 10, e.g. 11, e.g. 12, e.g. 13, e.g. 14, e.g. 15, e.g. 16, e.g. 17, e.g. 18, e.g. 19, e.g. 20, e.g. 21, e.g. 22, e.g. 23, e.g. 24, e.g. 25, e.g. 26, e.g. 27, e.g. 28, e.g. 29, e.g. 30, e.g. 35, e.g. 40, e.g. 45, e.g. 50, e.g. 60, e.g. 70, e.g. 80, e.g. 90, such as 100.

The present invention provides oligos which combine high affinity and specificity for their target RNA molecules with the ability to recruit RNAseH to an extend that makes them useful as antisense therapeutic agents. The oligos may be composed entirely of oxy-LNA monomers or they may be composed of both oxy and non-oxy-LNA monomers.

When both oxy-LNA and non-oxy-LNA monomer(s) are present in the oligo, the RNAseH recruiting characteristics of the chimeric oligo may be similar to, or different from, that of

the corresponding oxy-LNA oligo. Thus, in one aspect of the invention, non-oxy-LNA monomer(s) is/are used in such a way that they do not change the RNAseH recruting characteristics of the oxy-LNA/non-oxy-LNA chimeric oligo compared to the corresponding all oxy-LNA oligo. In another aspect of the invention the non-oxy-LNA monomer(s) is/are used purposely to change the RNAseH recruiting characteristics of an oxy-LNA oligo, either increasing or decreasing its efficiency to promote RNAseH cleavage when hybridised to its complementary RNA target compared to the corresponding all oxy-LNA oligo.

When both oxy-LNA and non-oxy-LNA monomer(s) is/are present in the oligo, the ability of the chimeric oligo to discriminate between its complementary target RNA and target RNAs containing one or more Watson-Crick mismatches may be different from the ability of the corresponding all oxy-LNA oligo to discriminate between its matched and mismatched target RNAs. For instance, the ability of an oxy-LNA oligo to discriminate between a complementary target RNA and a single base mismatched target RNA can be enhanced by incorporating non-oxy-LNA monomer(s), such as for instance DNA, RNA, thio-LNA or amino-LNA, either at, or close to, the mismatched position as described in applicant's Danish patent application entitled "Metod of increasing the specificity of oxy-LNA oligonuclotides" filed on the same day as the present application. Thus, in another aspect of the invention non-oxy-LNA monomer(s) is/are used purposely to construct an oxy-LNA/non-oxy-LNA oligo which exhibit increased specificity but unaltered RNAseH recruiting characteristics compared to the corresponding all oxy-LNA oligo. In another aspect of the invention the non-oxy-LNA monomer(s) is/are used purposely to construct an oxy-LNA/non-oxy-LNA oligo which exhibit both increased specificity and altered RNAseH recruiting characteristics compared to the corresponding all oxy-LNA oligo

Additionally, the oligonucleotide of the present invention may be conjugated with compounds selected from proteins, amplicons, enzymes, polysaccharides, antibodies, haptens and peptides.

Examples

Example 1: LNA containing oligonucleotides recruit RNase H

Two 41-mer oligonucleotides, that make up a linearised double-stranded template for subsequent T7 polymerase run-off transcription, were used to obtain target RNA corresponding to the following 15mer oligonucleotides:

DNA control; 5'- gtgtccgagacgttg-3'
phosphorothioate control; 5'-*gtgtccgagacgttg*-3'
LNA gab-mer; 5'-GTGTccgagaCGTTG-3' (LNA in capital letters, DNA is small letters) and
LNA-mix-mer; 5'-gTgTCCgAgACgTTg-3' (LNA in capital letters, DNA is small letters)

In the 5'end of the sense template strand, the promoter sequence for T7 polymerase recognition and initiation of transcription were contained, followed by the DNA sequence coding for the target-RNA sequence. The two complementary oligonucleotides were heated to 80°C for 10min to produce the linearised double-strand template. A 20µl in vitro transcription reaction containing 500µM each of ATP, GTP and CTP, 12µM of UTP, approx. 50µCi of α-^{32}P UTP, 1 x transcription buffer (Tris-HCl, pH 7.5), 10mM dithiotretiol, 1% BSA, 20 U of RNasin ribonuclease inhibitor, 0.2 µl template and 250 units T7 RNA polymerase. The inclusion of RNasin inhibitor was to prevent degradation of the target-RNA from ribonucleases. The reactions were carried out at 37°C for 2h to produce the desired 24mer ^{32}U-labelled RNA run-off transcript. For target RNA purification, 1.5µl (1.5 Units) of DNase I was added to the RNA which was resolved in a 15% polyacrylamide gel containing 7M urea and the correctly sized fragment was excised from the gel, dispensed in elution buffer (0.1% SDS, 0.5M ammonium acetate, 10mM Mg-acatate) and incubated at room temperature overnight. The target RNA sequence was then purified via ethanol precipitation, the supernatants filtered through a Millipore (0.45m) and collected by ethanol precipitation. The pellets were diluted in TE-buffer and subsequently subjected to RNAse H digestion assay. Herein, the decrease of intact substrate, *i.e.* the 24-mer α-^{32}P UTP labelled target RNA sequence, was assayed over time as follows. The reactions were carried out in a total volume of 110µl and contained (added in the order mentioned): 1 x nuclease-free buffer (20mM Tris-HCl, pH 7.5, 40mM KCl, 8mM $MgCl_2$, 0.03 mg/ml BSA), 10mM dithiotretiol, 4% glycerol, 100nM of oligonucleotide, 3 Units RNasin inhibitor, labelled target RNA strand and 0.1 U of RNase H. An excess of oligonucleotide was added to each reaction to ensure full hybridisation of the RNA target sequences. Two

negative controls were also included and were prepared as above but (1) without any oligonucleotide, or (2) without RNase H added to the reaction mixture. All the reactions were incubated at 37°C. At time points 0, 10, 20, 40 and 60 min., 10µl aliquots were taken and immediately added to ice-cold formamide loading buffer to quench the reaction and stored at –20°C. The samples were heated to 85°C for 5 min. prior to loading and running on a 15% polyacrylamide gel containing 7M urea. The gels were vacuum dried and exposed to autoradiographic films over night and subsequently subjected to densitometric calculations using the Easy Win imaging software (Hero Labs). The volume density of intact target RNA were calculated in each lane with correction for background. The volume density for the time zero sample was set as reference value for each incubation. Relative values for the other time-points samples in the corresponding incubation were calculated based on these reference values.

Brief description of figures

Figure 1 shows the results of the RNase H experiments. As expected the control DNA and phosphorothioate oligonucleotides both recruit RNAse H very efficiently. Also, as expected the LNA oligonucleotide which contains a contiguous stretch of six DNA monomers in the middle (LNA gab-mer) recruits RNAse H efficiently. Surprisingly, however, the LNA mix-mer which contains only single DNA monomers interdispersed between LNA monomers also recruits RNAse H. We conclude that the activity of RNase H is not contingent on a contiguous stretch of DNA or phosphorothioated bases when LNA is used as a component of the oligonucleotide.

Claims

1. A method for degrading RNA comprising providing a high affinity oligonucleotide which recruits RNaseH when hybridised to an RNA target sequence, wherein said high affinity oligonucleotide consists of oxy-LNA monomers exclusively.

2. A method for degrading RNA comprising providing a high affinity oligonucleotide which recruits RNaseH when hybridised to an RNA target sequence, wherein said high affinity oligonucleotide consists of both oxy-LNA and non-oxy-LNA monomers and wherein there are at the most five contiguous non-oxy-LNA monomers at any given position in the oligonucleotide.

3. A method for degrading RNA comprising providing a high affinity oligonucleotide which recruits RNaseH when hybridised to an RNA target sequence, wherein said high affinity oligonucleotide consists of both oxy-LNA and non-oxy-LNA monomers and wherein none of the non-oxy-LNA monomers are located adjacent to each other.

4. A method according to any of claims 2 or 3, wherein the presence of the non-oxy-LNA monomer(s) in the oxy-LNA/non-oxy-LNA oligo does not change the RNAseH recruiting characteristics of the oligo compared to the corresponding oxy-LNA oligo.

5. A method according to claim 4, wherein the presence of the non-oxy-LNA monomer(s) in the oxy-LNA/non-oxy-LNA oligo modifies the RNAseH recruiting characteristics of the oligo compared to the corresponding oxy-LNA oligo.

6. A method according to claim 5, wherein the presence of the non-oxy-LNA monomer(s) in the oxy-LNA/non-oxy-LNA oligo either enhances or reduces the ability of the oligo to recruit RNAseH compared to the corresponding oxy-LNA oligo.

7. A method according to any of the claims 2 to 6 wherein the presence of the non-oxy-LNA monomer(s) in the oxy-LNA/non-oxy-LNA oligo increases the ability of the oligo to discriminate between its complementary target RNA and target RNAs containing one or more Watson-Crick mismatches compared to the corresponding oxy-LNA oligo.

8. A method according to any of the previous claims wherein the oligonucleotide is characterised by the general formula

$$5'\text{-}[X_mY_nX_p]_q\text{-}3'$$

wherein X is oxy-LNA and Y is non-oxy-LNA, wherein m and p are integers from 0 to 30, n is an integer from 0 to 5 and q is an integer from 1 to 10.

9. A method according to claim 2-8, wherein the non-oxy-LNA monomer(s) is/are deoxyribonucleotide(s).

10. A method according to claim 9, wherein the deoxyribonucleotide is modified at the 2'-position in the ribose.

11. A method according to claim 10, wherein the 2'-modification is a hydroxyl, 2'-O-methyl, 2'-fluoro, 2'-trifluoromethyl, 2'-O-(2-methoxyethyl), 2'-O-aminopropyl, 2'-O-dimethylamino-oxyethyl, 2'-O-fluoroethyl or 2'-O-propenyl.

12. A method according to claim 11, wherein the modification also involves the 3' position, preferably modifications that links the 2'- and 3'-position in the ribose.

13. A method according to claim 12, wherein the modification also involves the 4' position, preferably modifications that links the 2'- and 4'-position in the ribose.

14. A method according to claim 13, wherein the modification is selected from the group consisting of a 2'-4' link being a $-CH_2-S-$, $-CH_2-NH-$, or $-CH_2-NMe-$ bridge.

15. A method according to any of the claims 9 to 14, wherein the nucleotide has the α-D-ribo, β-D-xylo, or α-L-xylo configuration.

16. A method according to any of the claims 9 to 15, wherein either all or some of the oxy-LNA monomers or all or some of the non-oxy-LNA monomer(s) or all or some of both the oxy-LNA monomers and non-oxy-LNA monomer(s) contain a 3'- or 5'- modification that results in an internucleoside linkage other than the natural phosphorodiester linkage.

17. A method according to claim 16, wherein the modification is selected from the group consisting of -O-P(O)$_2$-O-, -O-P(O,S)-O-, -O-P(S)$_2$-O-, -NR^H-P(O)$_2$-O-, -O-P(O,NR^H)-O-, -O-PO(R")-O-, -O-PO(CH$_3$)-O-, and -O-PO(NHR^N)-O-, where R^H is selected form hydrogen and C_{1-4}-alkyl, and R" is selected from C_{1-6}-alkyl and phenyl.

18. A method according to any of the preceding claims, wherein the incorporation of the at least one non-oxy-LNA monomer changes the affinity of the resulting oligo towards its complementary nucleic acid compared to the affinity of the all-oxy-LNA oligo by a ΔT_m of no more than ± 5°C.

19. A method according to claim 18, wherein the affinity is changed by no more than ± 10°C.

20. A method according to any of claims 18 or 19, wherein at least two non-oxy-LNA monomers containing either the same or different nucleobases at the 1'-position and being identical at all other positions are used.

21. A method according to any of claims 18 or 19, wherein at least two non-oxy-LNA monomers containing either the same or different nucleobases at the 1'-position and being non-identical in at least one other position are used.

22. A oligomer according to any of the previous claims, wherein said oligomer is used as a therapeutic compound, e.g. as an antisense compound.

23. An oligomer as defined in any of the previous claims, which is conjugated with compounds selected from proteins, amplicons, enzymes, polysaccharides, antibodies, haptens, and peptides.

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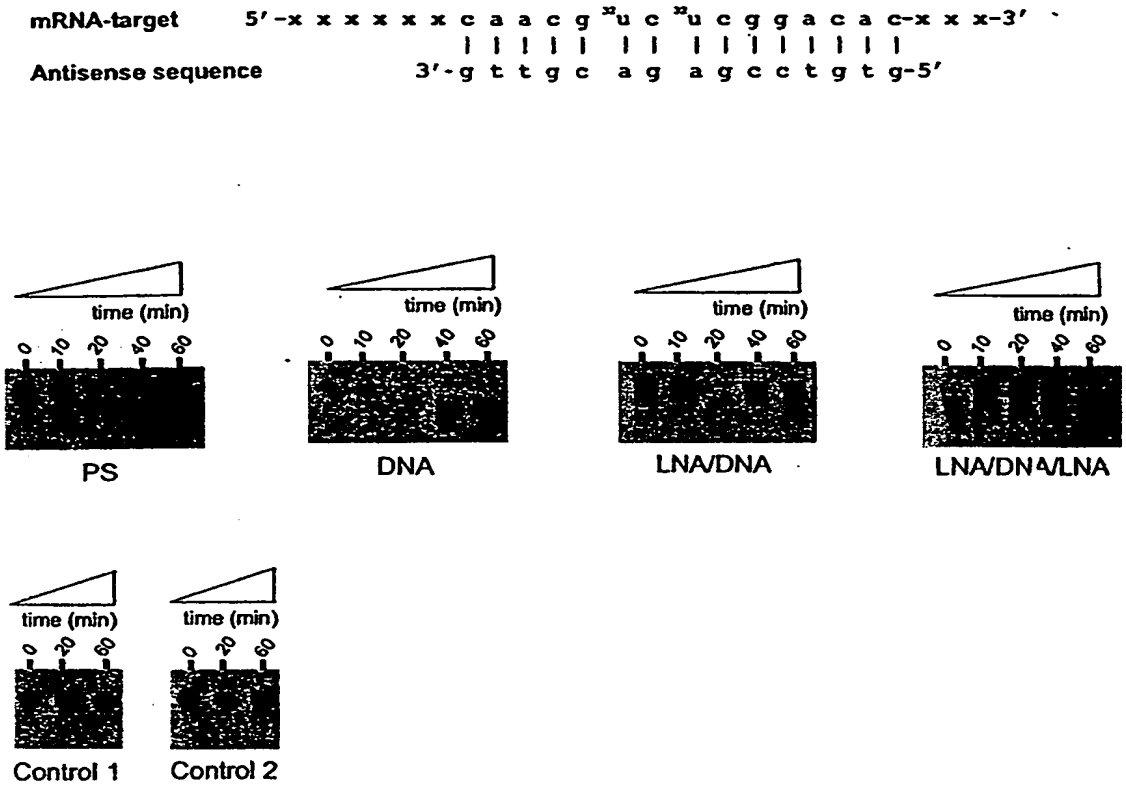
Figure 1.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Antisense sequence 3'-g t t g c a g a g c c t g t g-5'
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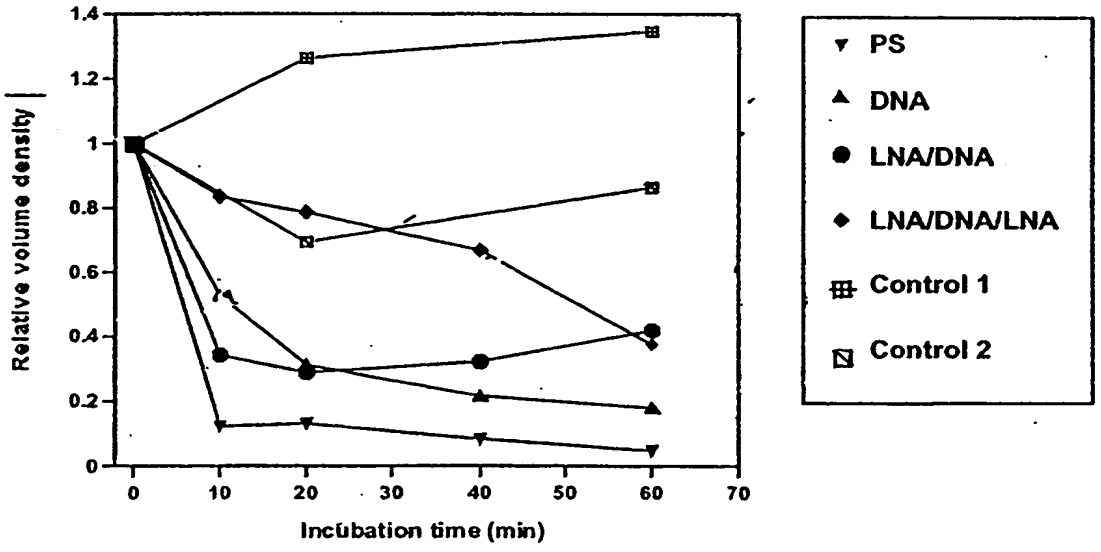
LNA/DNA
LNA/DNA/LNA
0 20 60
Control 1
Control 2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PS
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LNA/DNA
LNA/DNA/LNA
Control 1
Control 2

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α-L-RNA (α-L-*ribo* Configured RNA): Synthesis and RNA-Selective Hybridization of α-L-RNA/α-L-LNA Chimera

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Abstract—Synthesis of the novel α-L-ribofuranosyl phosphoramidite derivative **7** was accomplished via the α-L-ribofuranosyl thymine nucleoside **4**. Amidite **7** was used in automated syntheses of chimeric oligonucleotides composed of mixtures of the novel α-L-RNA nucleotide monomer ($^{\alpha L}$T, α-L-*ribo* configured RNA), and DNA, LNA (T^L, locked nucleic acid) or α-L-LNA ($^{\alpha L}T^L$, α-L-*ribo* configured locked nucleic acid) nucleotide monomers. For α-L-RNA/DNA and α-L-RNA/α-L-LNA chimeras, RNA-selective hybridization was obtained, for α-L-RNA/α-L-LNA chimera we found increased binding affinity compared to the corresponding DNA:RNA reference duplex. In addition, α-L-RNA/α-L-LNA chimera displayed significant stabilization towards 3′-exonucleolytic degradation. These results indicate that α-L-RNA/α-L-LNA chimeras deserve further evaluation as antisense molecules.

In the antisense therapeutic strategy, chemically modified oligonucleotides are administered in order to specifically inhibit the translation of disease-related mRNA sequences by duplex formation. Essential requirements for therapeutic efficiency of antisense oligonucleotides include good aqueous solubility, resistance toward enzymatic degradation, and high binding affinity and specificity towards the RNA target strand. Accordingly, synthesis of a large number of chemically modified oligonucleotides has been accomplished.[1] The unprecedented thermal stability of duplexes involving LNA (locked nucleic acid, β-D-*ribo* isomer, Fig. 1)[1b,2,3] has prompted us to study the properties of various LNA stereoisomers, including α-L-LNA (α-L-*ribo* configured LNA, Fig. 1).[1b,3–5] Very efficient recognition of single-stranded DNA and RNA has been demonstrated not only for LNA but also for α-L-LNA.[2–5]

Although significantly less stabilizing than LNA or α-L-LNA, 2′-*O*-alkyl-RNAs[6–8] are presently among the preferred nucleotide modifications in antisense oligonucleotides. The fact that 2′-*O*-alkyl-RNA/RNA duplexes[9] and LNA/RNA duplexes[2f] are not substrates for the RNA-cleaving enzyme RNase H limits the use of fully modified 2′-*O*-alkyl-RNA and fully modified LNA to RNase H independent antisense applications, for example, as steric blockers, as agents interfering with double-stranded RNA targets, or as end-gaps in gapmer[9] antisense oligonucleotides. However, high binding affinity and efficient mis-match discrimination are of utmost importance for any antisense application, as is

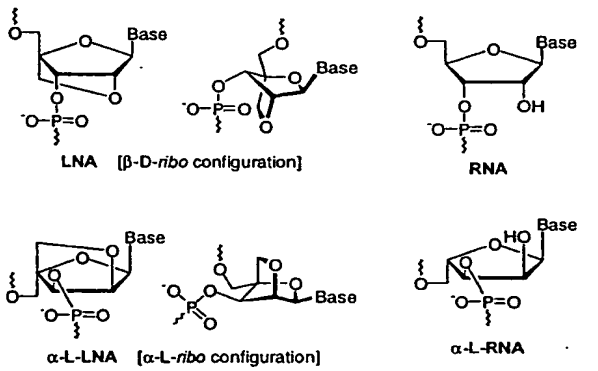

Figure 1. Structures of LNA and α-L-LNA nucleotide monomers and sketches of their locked *N*-type (C3′-*endo*, ^3E) furanose conformations.[5] Also shown are structures of RNA and α-L-RNA nucleotide monomers. In Table 1, the notations $^{\alpha L}$T (α-L-*ribo* configured RNA), T^L (LNA) and $^{\alpha L}T^L$ (α-L-LNA) are used for the monomers shown above (base = thymin-1-yl).

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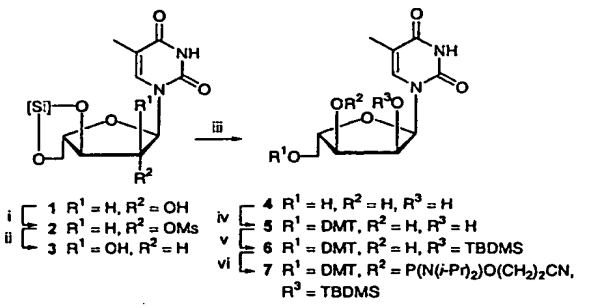

Scheme 1. Synthesis of the α-L-ribofuranosyl thymine nucleoside **4** and the phosphoramidite derivative **7**: (i) MsCl, pyridine (84%); (ii) aq NaOH, ethanol; (iii) aq NaOH, ethanol, 65 °C (59%, two steps); (iv) DMTCl, pyridine (93%); (v) TBDMSCl, imidazole, pyridine (45%); (vi) $NC(CH_2)_2OP(Cl)N(i\text{-}Pr)_2$, $EtN(i\text{-}Pr)_2$, CH_2Cl_2 (73%). [Si] = 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl. DMT = 4,4′-dimethoxytrityl.

the opportunity to fine-tune these parameters, for example, by combining nucleotide monomers of different chemical structures. This report is focused on the potential of modulating RNA-recognition by use of the novel α-L-RNA (α-L-*ribo* configured RNA, Fig. 1) with special focus on α-L-RNA/DNA and α-L-RNA/α-L-LNA chimeras.

Starting from L-arabinose, the known nucleoside **1**[10] was synthesized in five steps. Mesylation of compound **1** in pyridine gave compound **2** in an acceptable yield (84%). Subsequent treatment with aq NaOH in ethanol afforded the *ribo*-configured nucleoside **3**, presumably via an O2′,C2-anhydronucleoside intermediate. Subsequent heating of the reaction mixture for 18 h effected removal of the 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl protecting group and 1-(α-L-ribofuranosyl)thymine (**4**) was isolated in 59% yield (from **2**). The NMR data of **4** were identical to those of its enantiomer, 1-(α-D-ribofuranosyl)thymine.[11] Nucleoside triol **4** was selectively DMT-protected in 93% yield at the primary hydroxy group by reaction with 1.5 equiv DMTCl in pyridine to give derivative **5**, which after reaction with 3.0 equiv TBDMSCl in pyridine in the presence of imidazole afforded a mixture of the 2′-*O*- and 3′-*O*-silylated products. Separation of these by column chromatography (2.0–6.0% acetone/0.5% pyridine/97.5–93.5% dichloromethane; v/v/v) furnished nucleoside **6** in 45% yield. Nucleoside **6** was dissolved in anhydrous dichloromethane in the presence of *N*,*N*-diisopropylethylamine and 2-cyanoethyl *N*,*N*-diisopropylphophoramidochloridite to give phosphoramidite **7** in 73% yield (Scheme 1).

The oligomers (Table 1) used in this study were synthesized on an automated DNA synthesizer using the phosphoramidite approach.[12] The α-L-ribofuranosyl phosphoramidite building block **7** was used for the synthesis of the α-L-RNA oligomers **11**, **12** and **16–20**. The stepwise coupling yield for amidite **7** was approximately 98% (20 min coupling time; 1*H*-tetrazole as activator) using procedures described previously.[4a] After detritylation, cleavage from the solid support and deacylation were effected using 40% aqueous methylamine (10 min, 55 °C). After cooling to −18 °C, the solid support was removed (centrifugation), washed [2 × 0.25 cm^3; $EtOH/CH_3CN/H_2O$ (3:1:1, v/v/v)], and the combined liquid phase evaporated to dryness under reduced pressure. The residue was desilylated using a method described previously[13] during 20 h at 55 °C. Desilylation of the oligomers was incomplete when using milder desilylation conditions as revealed by MALDI-MS analysis. Standard conditions of the synthesizer were used for incorporation of DNA monomers whereas the incorporation of LNA or α-L-LNA monomers followed procedures described previously.[2a,2b,4a] Analysis by capillary gel electrophoresis verified the purity of the novel α-L-RNA oligomers **11**, **12** and **16–20** as being >90%, whereas MALDI-MS analysis confirmed their compositions.[14] The reference DNA oligomers **8** and **13**,[2a,4b] the LNA oligomers **9** and **14**,[2a,2b] and the α-L-LNA oligomers **10** and **15**[4b,4c] have been prepared and studied previously.

Table 1. Melting temperatures (T_m values) towards complementary single-stranded DNA and RNA targets obtained as the maximum of the first derivative of the melting curve (A_{260} vs temperature) in medium salt buffer (10 mM sodium phosphate, 100 mM sodium chloride, 0.1 mM EDTA, pH 7.0) using 1.5 μM concentrations of the two complements[a]

Entry	Description of oligomers	Sequence of oligomers	DNA complement T_m (°C)	RNA complement T_m (°C)
1	DNA reference	**8**: GTGATATGC	30	28
2	LNA/DNA	**9**: $GT^LGAT^LAT^LGC$	44	50
3	α-L-LNA/DNA	**10**: $G(^{\alpha L}T^L)GA(^{\alpha L}T^L)A(^{\alpha L}T^L)GC$	37	45
4	α-L-RNA/DNA	**11**: $GTGA(^{\alpha L}T)ATGC$	26	28
5	α-L-RNA/DNA	**12**: $G(^{\alpha L}T)GA(^{\alpha L}T)A(^{\alpha L}T)GC$	<5	12
6	DNA reference	**13**: T_{10}	20	19
7	LNA	**14**: $(T^L)_9T$	80	71
8	α-L-LNA	**15**: $(^{\alpha L}T^L)_9T$	63	66
9	α-L-RNA	**16**: $(^{\alpha L}T)_9T$	<5	<5
10	α-L-RNA/LNA	**17**: $(^{\alpha L}T)_4(T^L)_4(^{\alpha L}T)T$	<5	27
11	α-L-RNA/LNA	**18**: $[(^{\alpha L}T)(T^L)]_4(^{\alpha L}T)T$	<5	<5
12	α-L-RNA/α-L-LNA	**19**: $(^{\alpha L}T)_4(^{\alpha L}T^L)_4(^{\alpha L}T)T$	<5	29
13	α-L-RNA/α-L-LNA	**20**: $[(^{\alpha L}T)(^{\alpha L}T^L)]_4(^{\alpha L}T)T$	<5	27

[a]All oligomers are depicted with the 5′-end positioned to the left. A = adenine monomer, C = cytosine monomer, G = guanine monomer, T = thymine monomer; A, C, G and T are DNA monomers, that is, 2′-deoxy-β-D-ribofuranosyl derivatives. See Fig. 1 for structures of the T^L, $^{\alpha L}T^L$, and $^{\alpha L}T$ monomers (Base = thymin-1-yl).

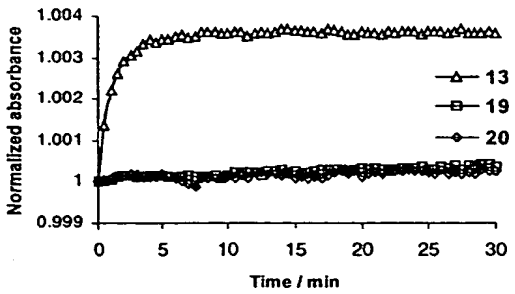

Figure 2. Time course of snake venom phosphodiesterase digestion of the DNA reference **13** and the α-L-RNA/α-L-LNA chimeras **19** and **20**. A solution of the oligomers (~0.2 OD) in 2 mL of a buffer (0.1 M Tris–HCl; pH = 8.6; 0.1 M NaCl; 14 mM $MgCl_2$) was digested with 1.2 U snake venom phosphodiesterase [30 μL of a solution in the following buffer: 5 mM Tris–HCl; pH = 7.5; 50% glycerol (v/v)] at 25 °C.

Results from hybridization experiments (T_m values) toward single-stranded DNA and RNA complements are depicted in Table 1. In entries 1–5, different variations of a 9-mer mixed-base sequence are studied. Introduction of three thymine LNA monomers (**9**)[2a,2b] or three α-L-LNA monomers (**10**)[4c] significantly improves the thermal stability towards both DNA and RNA when compared to the results obtained with the corresponding DNA reference **8**. Both **9** and **10** display a weak RNA selectivity as witnessed by the slightly lower thermal stabilities of the duplexes involving the DNA complement. Results for the two α-L-RNA/DNA chimeras **11** and **12** are despicted in entries 4 and 5. Incorporation of one α-L-RNA nucleotide leads to unchanged (toward RNA) or reduced (toward DNA, $\Delta T_m = -4$ °C) thermal stability when compared to the DNA reference **8**. When three α-L-RNA monomers are incorporated (oligomer **12**), hybridization towards both DNA and RNA is adversely influenced, most so, however, toward DNA.

Various combinations of the different monomers in a homothymine 10-mer context are evaluated in the second series of oligomers (Table 1, entries 6–13). As reported earlier, the (almost) fully modified LNA and α-L-LNA oligomers **14** and **15**, respectively, indeed display very efficient hybridization towards both DNA and RNA.[2a,3b,3c] In contrast, a melting point was not obtained for the corresponding α-L-RNA **16** neither toward DNA nor RNA. Exchange of four α-L-RNA monomers of **16** with LNA monomers gave the α-L-RNA/LNA chimeras **17** and **18**. With four consecutive LNA monomers (**17**), no hybridization toward the DNA complement was detected. However, a T_m value of 27 °C toward the RNA complement was observed. This strong RNA selectivity is thought to be caused by hybridization of the LNA segment (-T^L_4-) with the RNA but not with the DNA complement, as supported by hybridization results reported earlier for a T^L_5T oligomer.[2a] A comparable RNA selectivity has not been observed neither for longer homothymine sequences (e.g., **14**) nor for partly or fully modified LNAs with mixed base compositions.[2b,15] The α-L-RNA/LNA chimera **18** with alternating α-L-RNA and LNA monomers hybridizes neither with the DNA nor the RNA complement. The two α-L-RNA/α-L-LNA chimeras **19** and **20** containing four consecutive α-L-LNA monomers and alternating α-L-RNA and α-L-LNA monomers, respectively, display very similar binding properties. Thus, T_m values of 29 °C and 27 °C for **19** and **20**, respectively, were observed toward the RNA target but no T_m values toward the DNA target. Of utmost importance for antisense applications are not only binding affinity but also base-pairing selectivity. It is therefore encouraging that oligomers **19** and **20** both display satisfactory discriminatory behavior towards an RNA target with one mis-matched base [r(A_7CA_6) target: T_m < 5 °C]. However, more experiments are needed in order to fully understand the binding interactions between RNA and α-L-RNA/α-L-LNA chimera.

The stability of α-L-RNA/α-L-LNA chimera toward 3′-exonucleolytic degradation in vitro was evaluated using snake venom phosphodiesterase (SVPDE).[16] During SVPDE digestion of unmodified oligonucleotides, for example the DNA reference **13** (see Fig. 2), the absorbance at 260 nm rapidly increases due to conversion into the nucleoside constituents.[16] In contrast, the α-L-RNA/α-L-LNA chimeras **19** and **20** are both very significantly stabilized toward degradation by SVPDE (Fig. 2; no significant hyperchromicity observed). These qualitative experiments indicate that α-L-RNA/α-L-LNA chimera, like α-L-DNA,[17] α-L-LNA,[18] and DNA/α-L-LNA chimera,[18] are significantly protected toward 3′-exonucleolytic degradation.

The results reported herein suggest that further studies should be performed in order to evaluate the full potential of α-L-RNA/α-L-LNA chimeras as antisense molecules. If the pronounced RNA selectivity obtained for **20** (composed of alternating α-L-RNA and α-L-LNA monomers) turns out to be a general feature of α-L-RNA/α-L-LNA chimeras, one may envision reduced toxicity and improved specificity compared to the current antisense molecules which are known to be able also to hybridize toward DNA targets. A similarily pronounced RNA selectivity has been reported for a few other oligonucleotide analogues, for example, β-L-DNA,[19] arabinonucleic acids,[20] 2′-*O*,3′-*C*-linked bicyclic oligonucleotides,[21] and α-D-LNA.[22] However, their usefulness as antisense molecules is hampered either by comparatively low binding affinity toward RNA[19,20] or the necessity of using fully modified oligomers in order to obtain efficient RNA binding.[21,22] It is therefore important that the α-L-RNA/DNA chimera **11** retains the ability to hybridize to RNA and that the α-L-RNA/α-L-LNA chimera **20**, with alternating α-L-RNA and α-L-LNA monomers, displays increased binding affinity towards RNA (ΔT_m value = +8 °C compared to the DNA reference **13**). It is striking that both DNA/α-L-LNA and α-L-RNA/α-L-LNA chimeras display increased binding affinity toward RNA which stresses the flexibility and power of these LNA-type chimeric oligonucleotides.

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Expanding the design horizon of antisense oligonucleotides with alpha-L-LNA

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ABSTRACT

Oligonucleotides containing Locked Nucleic Acids (LNA) to various extents and at various positions were evaluated for antisense activity, RNase H recruitment, nuclease stability and thermal affinity. In this work, two different diastereoisomers of LNA were studied: the beta-D-LNA and the alpha-L-LNA (abbreviated as β-D-LNA and α-L-LNA). Our findings show that the best antisense activity with 16mer gapmers containing β-D-LNA (oligonucleotides containing consecutive segments of LNA and DNA with a central DNA stretch flanked by two LNA segments, LNA–DNA–LNA) is found with gap sizes between 7 and 10 nt. The optimal gap size is motif-dependent, and requires the right balance between gap size and affinity. Compared to β-D-LNA, α-L-LNA shows superior stability against a 3′-exonuclease. The design possibilities of α-L-LNA were explored for different gapmers and other designs, collectively called chimeras. The placement of α-L-LNA in the junctions or in the flanks resulted in potent antisense oligonucleotides. Moreover, different chimeras with an alternate composition of DNA, α-L-LNA and β-D-LNA were evaluated in terms of antisense activity and RNase H recruitment. Chimeras with an interrupted DNA stretch with α-L-LNA still recruit RNase H and show good levels of antisense activity, while the same design with β-D-LNA results in a drop in antisense potency. Our findings indicate that α-L-LNA is a powerful and versatile nucleotide analogue for designing potent antisense oligonucleotides.

INTRODUCTION

The antisense strategy is based on the inhibition of gene expression as a result of the Watson–Crick complementary hybridization of oligonucleotides to single stranded RNA (1), thereby interfering with the expression of the encoded protein (2). Different mechanisms (3) have been described to account for the antisense activity, such as steric blockage of the translational machinery and RNase H based cleavage of the mRNA.

So far, phosphorothioate oligonucleotides have been the most widely used class of antisense compounds, and several phosphorothioate oligonucleotides are currently being tested in clinical trials. Despite their indisputable therapeutic potential, they have been associated with non-specific effects caused by interactions with intracellular and cell-surface proteins and non-specific cleavage of unintended targets (4).

Numerous sugar, base and backbone chemical modifications have been investigated to improve the binding affinity and increase the nuclease stability of antisense oligonucleotides. Modification of the 2′-*O* position of the ribose moiety has been the focus of much research (5). In general, 2′-*O* modified oligonucleotides cannot act as a substrate for RNase H, which so far seems to be the single important mechanism for control of gene expression. This potential drawback in the application of modified oligonucleotides for down-regulation has been remedied by the use of chimeric oligonucleotides (6), the so-called gapmers, which comprise a central stretch of RNase H recruiting nucleotides (typically phosphorothioates) flanked by modified nucleotides.

Much of the recent interest has been focused on the preparation of conformationally restricted derivatives. Beta-D-LNA (β-D-LNA, Locked Nucleic Acid), first described by Wengel and co-workers (7,8) and by Imanishi and co-workers (9), contains a methylene 2′-*O*, 4′-*C* linkage (Fig. 1). This bridge reduces the conformational flexibility and confers a RNA-like C3′-endo conformation to the sugar moiety of the nucleotide (10). This greatly enhances affinity towards DNA and RNA targets (ΔT_m values from 4.0 to 9.3°C per introduced LNA monomer compared to unmodified duplexes) (11).

In 2002, Erdmann and co-workers (12) presented a systematic study on the nuclease stability and RNase H recruiting of β-D-LNA containing oligonucleotides using 18mers and concluded that a gap of 7 or 8 nt DNA is necessary for activation. The gapmers further exhibited increased stability against nucleases; as little as three β-D-LNAs at each end of the oligonucleotide increased the stability in human serum more than 10-fold.

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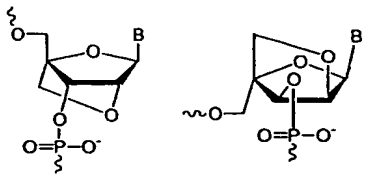

Figure 1. Chemical structures of β-D-LNA and α-L-LNA.

The unprecedented thermal stability of duplexes involving β-D-LNA prompted researchers to study the properties of various LNA stereoisomers (13,14), especially alpha-L-LNA (α-L-LNA) (15–18) (Fig. 1). It is well established that β-D-LNA is locked in a N-type conformation and thus is yielding an A-form duplex with complementary DNA (17,19) and an almost canonical A-form (the natural form of double stranded RNA) with complementary RNA (10). Duplexes between α-L-LNA and DNA adopt a B-form (20) (the natural form of double stranded DNA), whereas duplexes α-L-LNA:RNA generate an intermediate structure between A and B form (16), which is structurally closer to the natural substrate of RNase H and thus is able to partially elicit RNase H activity (18). α-L-LNA has also been reported to confer increased stability towards nucleases (18).

Here we report the biochemical and biophysical properties of oligonucleotides containing α-L-LNA, β-D-LNA and chimeras with all three β-D-LNA, α-L-LNA and DNA. In particular, a systematic evaluation of different chimeric oligonucleotides of different size, design and load of LNA, in terms of RNase H activity, nuclease stability and down-regulation of target mRNA was carried out. We confirm the results of Erdmann and co-workers (12) and further complement them with more data regarding β-D-LNA.

MATERIALS AND METHODS

Monomer synthesis

The α-L-LNA monomers were prepared following published procedures (18) with the exception of the final phosphitylation step which was performed according to Pedersen *et al.* (21). The β-D-LNA monomers were obtained from Proligo (Boulder, CO).

Oligonucleotide synthesis

Oligonucleotides were synthesized using the phosphoramidite approach on an Expedite 8900/MOSS synthesizer (Multiple Oligonucleotide Synthesis System) at 1 μmol scale. At the end of the synthesis (DMT-on), the oligonucleotides were cleaved from the solid support using aqueous ammonia for 1–2 h at room temperature, and further deprotected for 4 h at 65°C. The oligonucleotides were purified by reverse phase HPLC (RP-HPLC). After the removal of the DMT-group, the oligonucleotides were characterized by AE-HPLC or RP-HPLC, and the molecular mass was further confirmed by ESI-MS.

UV-melting measurements

Melting curves were recorded with a Perkin Elmer UV/Vis spectrophotometer lambda 40 attached to a PTP-6 Peltier System. Oligonucleotides were dissolved in buffer (20 mM phosphate buffer, 100 mM NaCl, 0.1 nM EDTA, pH 7) using the two complementary strands at 1.5 μM and 1 cm path-length cells. Samples were denatured at 95°C for 3 min and slowly cooled to 20°C prior to measurements. Melting curves were recorded at 260 nm using a heating rate of 1°C/min, a slit of 2 nm and a response of 0.2 s. T_m values were obtained from the maxima of the first derivatives of the melting curves.

3′-Exonuclease stability study

Snake venom phosphodiesterase (SVPD, Amersham Biosciences) assays were performed using 26 μg/ml oligonucleotide, 0.3 μg/ml enzyme at 37°C in a buffer of 50 mM Tris–HCl, 10 mM $MgCl_2$, pH 8. The enzyme was shown to maintain its activity under these conditions for at least 2 h. Aliquots of the enzymatic digestion were removed at the indicated times, quenched by heat denaturation for 3 min and stored at –20°C until analysis by RP-HPLC.

S1 endonuclease stability study

S1 endonuclease (Amersham Biosciences) assays were performed using 1.5 μM oligonucleotide and 16 U/ml enzyme at 37°C in a buffer of 30 mM NaOAc, 100 mM NaCl, 1 mM $ZnSO_4$, pH 4.6. The enzyme was shown to maintain its activity under these conditions for at least 2 h. Aliquots of the enzymatic digestion were removed at the indicated times, quenched by lyophilization, and stored at –20°C until analysis by either RP-HPLC and ESI-MS or polyacrylamide electrophoresis.

Luciferase assay

The X1/5 HeLa cell line (ECACC Ref. No. 95051229) stably transfected with a Tet-Off luciferase system was used. In the absence of tetracycline, the luciferase gene is expressed constitutively. The expression was measured as light emission in a luminometer after addition of the luciferase substrate (luciferin). The X1/5 HeLa cell line was grown in Minimum Essential Medium Eagle (Sigma M2279) supplemented with 1× Non Essential Amino Acid (Sigma M7145), 1× Glutamax I (Invitrogen 35050–038), 10% FBS calf serum, 25 μg/ml Gentamicin (Sigma G1397), 500 μg/ml G418 (Invitrogen 10131–027) and 300 μg/ml Hygromycin B (Invitrogen 10687–010). The X1/5 HeLa cells were seeded at a density of 8000 cells/well in a white 96-well plate (Nunc 136101) the day before transfection. Before the transfection, the cells were washed once with OptiMEM (Invitrogen) followed by addition of 40 μl of OptiMEM with 2 μg/ml of Lipofectamine2000 (Invitrogen). The cells were incubated for 7 min before addition of the oligonucleotides. Then 10 μl of oligonucleotide solutions were added and the cells were incubated for 4 h at 37°C and 5% CO_2. The cells were then washed once in OptiMEM and growth medium was added (100 μl). The luciferase expression was measured the day after. Luciferase expression was measured with the Steady-Glo luciferase assay system from Promega. 100 μl of the Steady-Glo reagent was added to each well and the plate was shaken for 30 s at 700 r.p.m. The plate was read in a

Luminoscan Ascent instrument from ThermoLab systems after 8 min of incubation to complete total lysis of the cells. Luciferase expression was measured as Relative Light Units per second (RLU/s). The data were processed with Ascent software (v2.6) and graphs were drawn with SigmaPlot2001. All data were normalized to mock levels. No significant cell death was observed by microscopy for the duration of the assay.

RNase H assay

The RNA was radiolabeled (^{32}P) at the 5′-end with T4 kinase (Invitrogen) following well-established procedures; 25 nM RNA was incubated in the presence of a 10-fold excess of various complementary oligonucleotides in 1× TMK-glutamate buffer [20 mM TrisOAc, 10 mM $Mg(OAc)_2$ and 200 mM potassium glutamate, pH 7.25] supplied with 1 mM DTT in a reaction volume of 40 µl. The reactions were pre-incubated for 3 min at 65°C followed by 15 min at 37°C before addition of RNase H (Promega); 0.2 U of RNase H was added, and samples were withdrawn (6 µl) to formamide dye (3 µl) on ice at the time points 0, 10, 20 and 30 min after RNase H addition; 3 µl of the 0, 10, 20 and 30 min samples were loaded on a 15% polyacrylamide gel containing 6 M urea and 0.9× Tris borate/EDTA buffer. The gel was 0.4 mm thick and run at 35 W for 2 h. The gel was dried for 60 min at 80°C, followed by overnight exposure on a Kodak phosphorscreen. The Kodak phosphorscreen was read in a Bio-Rad FX instrument and the result was analysed with Bio-Rad Quantity One software.

RESULTS AND DISCUSSION

β-D-LNA gapmers: evaluation of the optimal gap size

Our first goal was to determine the optimal DNA stretch (gap size) for a 16mer β-D-LNA/DNA chimera for best antisense activity, and correlate it with the corresponding affinity against the complementary DNA strand.

In a previous study, we tested different gapmers (16mers, 7 nt gap size) containing β-D-LNA against firefly luciferase mRNA by messenger walk screening, and observed moderate potencies for motifs 1, 2 and 3 in a dose–response manner.

The three different motifs of the mRNA of the firefly luciferase were targeted with 16mers with a PS gap and PO phosphate interlinkages in the β-D-LNA flanks. The DNA gap size varied from 7 to 10 nt with the β-D-LNA segment length adjusted accordingly to keep the overall length of the chimeric compound fixed (**1–12** in Table 1).

For all three studied motifs, it has been seen that the affinity drops with the reduction of LNA residues in the oligonucleotide and that the affinity is sequence dependent (Fig. 2). Gapmers (**1–4**) against motif 1 show a high effect in down-regulation with the increase in the gap size, with 10 nt being the best. This 10 nt gapmer (**4**) still presents a very high affinity (T_m 61.3°C). Gapmers (**5–8**) against motif 2 exhibit a reduced effect in down-regulation as the gap size increases, 7 nt (**5**) being the most potent one (T_m 52.4°C). For this motif, the increase in gap size results in a critical loss of affinity resulting in less potent oligonucleotides. For motif 3, the 9 nt gap (**11**) (T_m 58.0°C) proves to be the most potent gapmer.

For motif 1, for which the affinity remains high enough, a larger gap (10 nt over 7, 8 and 9 nt) is an advantage in terms of RNase H recruitment and thus antisense activity, whereas motif 2 evidences that if the affinity lies within a low range, the gap size is not so determinant. Motif 3 affinities are apparently around the critical limit range; therefore both affinity and gap size play a role in determining the activity of these gapmers. These three motifs clearly demonstrate that there is a delicate balance between affinity and optimal gap size. In our assay, all gapmers showed extensive RNase H recruitment (*vide infra*).

We also repeatedly observed for chimeric oligonucleotides containing β-D-LNA an overall trend of better down-regulation with an increase in the number of PS interlinkages for different gapmer designs. This is illustrated by motif 3 (gap PS) in Figure 2 compared to the 16mer (all PS) in Figure 3. Fully thiolated gapmers (**13–16**, all PS) perform better in the Luciferase assay than gapmers with a thiolated gap (**9–12**, gap PS), and than the gapmers with all PO interlinkages (data not shown). The much lower activity of the all PO gapmers could be due to their rapid degradation by nucleases (*vide infra*).

β-D-LNA gapmers: evaluation of the size and the gap size

The high affinity achieved by the introduction of β-D-LNA (**11**) should allow for the design of potent and short antisense

Table 1. Sequences of the oligonucleotides used in this study

	Sequence	Design	Tm (°C)
Motif 1: Gapmers			
1	GTCT$_t$t$_c$c$_c$c$_g$t$_g$CTCCA	Gap of 7	71.7
2	GTCT$_t$t$_c$c$_c$g$_t$t$_g$c$_t$TCCA	Gap of 8	68.0
3	GTC$_t$t$_t$c$_c$c$_g$t$_g$c$_t$TCCA	Gap of 9	65.0
4	GTC$_t$t$_t$c$_c$c$_g$t$_g$c$_t$t$_c$CCA	Gap of 10	61.3
Motif 2: Gapmers			
5	TCTTc$_t$t$_t$c$_a$a$_a$a$_a$TCTAT	Gap of 7	52.4
6	TCTTc$_t$t$_t$c$_a$a$_a$a$_a$t$_c$CTAT	Gap of 8	50.3
7	TCTt$_c$t$_t$t$_c$a$_a$a$_a$t$_c$CTAT	Gap of 9	46.2
8	TCTt$_c$t$_t$t$_c$a$_a$a$_a$t$_c$t$_c$TAT	Gap of 10	42.2
Motif 3: Gapmers			
9	TTCCg$_t$c$_a$t$_c$g$_t$TCTTT	Gap of 7	66.8
10	TTCCg$_t$c$_a$t$_c$g$_t$c$_t$CTTT	Gap of 8	63.4
11	TTCc$_g$t$_c$a$_t$c$_g$t$_c$CTTT	Gap of 9	58.0
12	TTCc$_g$t$_c$a$_t$c$_g$t$_c$t$_c$TTT	Gap of 10	54.7
13	T$_t$T$_t$C$_t$C$_g$t$_c$a$_t$c$_g$T$_c$T$_t$T$_t$T	Gap of 7	64.1
14	T$_t$T$_t$C$_t$C$_g$t$_c$a$_t$c$_g$t$_c$T$_t$T$_t$T	Gap of 8	61.3
15	T$_t$T$_t$C$_t$c$_g$t$_c$a$_t$c$_g$t$_c$T$_t$T$_t$T	Gap of 9	58.5
16	T$_t$T$_t$C$_t$c$_g$t$_c$a$_t$c$_g$t$_c$t$_c$T$_t$T$_t$T	Gap of 10	53.0
Motif 3: Short size gapmers (12 and 14mers)			
17	T$_t$C$_t$C$_g$t$_c$a$_t$c$_g$t$_c$T$_t$T	Gap of 8	57.0
18	T$_t$C$_t$c$_g$t$_c$a$_t$c$_g$t$_c$T$_t$T	Gap of 10	51.0
19	C$_t$C$_g$t$_c$a$_t$c$_g$T$_t$C$_t$T	Gap of 7	54.4
20	C$_t$C$_g$t$_c$a$_t$c$_g$t$_c$C$_t$T	Gap of 8	50.5
21	C$_t$c$_g$t$_c$a$_t$c$_g$t$_c$C$_t$T	Gap of 9	46.1
Motif 3: α-L-LNA gapmers			
22	TTCC$^{\alpha}$g$_t$c$_a$t$_c$g$_t$T$^{\alpha}$CTTT		64.8
23	T$^{\alpha}$T$^{\alpha}$C$^{\alpha}$C$^{\alpha}$g$_t$c$_a$t$_c$g$_t$T$^{\alpha}$C$^{\alpha}$T$^{\alpha}$T$^{\alpha}$T$^{\alpha}$		65.2
24	T$^{\alpha}$T$^{\alpha}$C$^{\alpha}$C$^{\alpha}$gtcatcgT$^{\alpha}$C$^{\alpha}$T$^{\alpha}$T$^{\alpha}$T$^{\alpha}$		68.6
25	T$^{\alpha}_s$T$^{\alpha}_s$C$^{\alpha}_s$C$^{\alpha}_s$g$_t$t$_c$a$_t$c$_g$T$^{\alpha}_s$C$^{\alpha}_s$T$^{\alpha}_s$T$^{\alpha}_s$T$^{\alpha}$		62.8
Motif 3: Chimeras			
26	TTCCg$_t$c$_a$A$_t$c$_g$TCTTT		67.0
27	TTCCg$_t$c$_a$A$^{\alpha}_s$t$_c$g$_t$TCTTT		68.4
28	TTCC$^{\alpha}$g$_t$c$_a$A$^{\alpha}_s$t$_c$g$_t$T$^{\alpha}$CTTT		67.3
29	TTCCg$_t$T$_c$a$_t$c$_g$T$_c$TTT		67.0
30	TTCCg$_t$T$^{\alpha}_s$c$_a$t$_c$g$_t$T$^{\alpha}_s$c$_t$TTT		66.3
31	T$^{\alpha}$T$^{\alpha}$C$^{\alpha}$C$^{\alpha}$g$_t$T$^{\alpha}_s$c$_a$t$_c$g$_t$T$^{\alpha}_s$c$_t$T$^{\alpha}$T$^{\alpha}$T$^{\alpha}$		60.3
32	T$_t$T$_t$C$_t$C$_g$t$_c$A$_t$T$_c$G$_g$T$_c$T$_t$T$_t$T		82.0
33	T$^{\alpha}_s$T$^{\alpha}_s$C$^{\alpha}_s$c$_g$t$_c$C$^{\alpha}_s$A$^{\alpha}_s$T$^{\alpha}_s$c$_g$t$_c$C$^{\alpha}_s$T$^{\alpha}_s$T$^{\alpha}_s$t		63.0
34	T$^{\alpha}_s$T$^{\alpha}_s$C$^{\alpha}_s$C$^{\alpha}_s$g$_t$T$^{\alpha}_s$C$^{\alpha}_s$A$^{\alpha}_s$T$^{\alpha}_s$C$^{\alpha}_s$g$_t$T$^{\alpha}_s$C$^{\alpha}_s$T$^{\alpha}_s$T$^{\alpha}_s$T$^{\alpha}$		76.0
Mismatch controls for motif 3			
35	TTCCg$_t$c$_t$a$_c$g$_t$TCTTT	2 mismatches	55.2
36	TTCCt$_t$t$_c$c$_t$c$_t$t$_t$TCTTT	3 mismatches	<20

The corresponding designs are also illustrated with drawings for the different gapmers and chimeras. Nomenclature for design column: line is DNA, the rectangle is β-D-LNA and the shaded rectangle is α-L-LNA residues. Upper case for LNA and lower case for DNA.

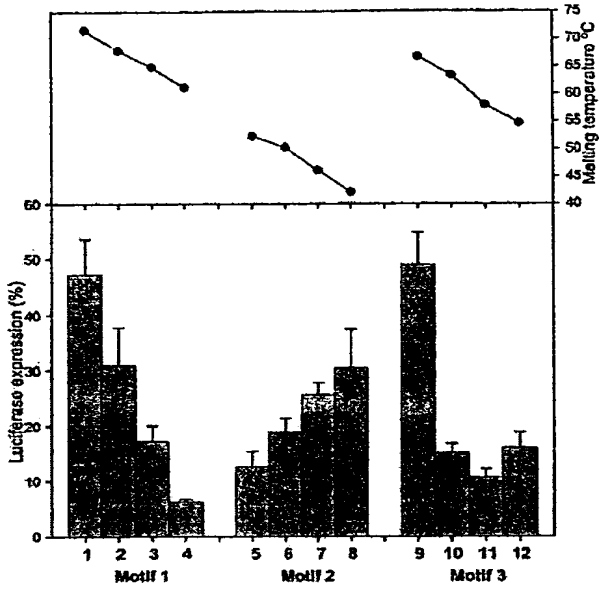

Figure 2. Down-regulation of Luciferase expression by gapmers (1–12) with PS in the gap and PO in the flanks containing an increasing gap size from 7 to 10 nt directed against three different motifs (1, 2 and 3) at 2 nM. The melting temperatures (T_m) for the different gapmers are also included in the graph.

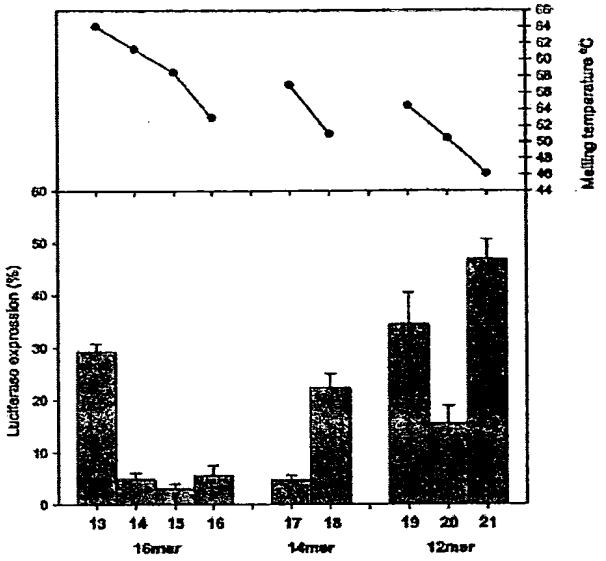

Figure 3. Down-regulation of Luciferase expression by fully thiolated oligonucleotides (all PS, 17–21) with reduced size and various gap sizes (12 and 14mers) at 2 nM. The 16mer (all PS, 13–16) is included as a reference. The melting temperatures (T_m) for the different gapmers are also included in the graph.

oligonucleotides (<15 nt). Therefore, we evaluated different gap sizes for shorter oligonucleotides, 12 and 14mers, in terms of antisense activity, and correlated the data with the corresponding affinity against a complementary DNA strand. All oligonucleotides were also tested for the ability to direct target RNA cleavage by RNase H. Different 14mers (**17**, **18**) and 12mers (**19**–**21**) against motif 3 (both ends-shortened oligonucleotides compared to the previously described 16mers) were prepared with all PS, and the DNA gap size varied with the β-D-LNA segment length adjusted accordingly to keep the overall length of the chimeric compound fixed.

As it can be appreciated from Figure 3, the balance between affinity and gap size seems for these 14 and 12mers to prefer a gap of 8 nt (**17** and **20**). For the 14mer, an increased gap (10 nt, **18**), and therefore a lower β-D-LNA substitution, corresponds to a decrease in affinity, which is reflected in a drop in antisense activity. Compared to the 16 and 14mers, the 12mers show both lower affinity and lower antisense activity, and present maximal activity for the 8 nt gap sized gapmer. As previously shown for the 16mers, a compromise between gap size and affinity determines the optimal gap size for a chimeric oligonucleotide containing β-D-LNA. Again in our assay, all gapmers showed extensive RNase H recruitment.

In order to prove that down-regulation is specific for a sequence-depending mechanism, we further tested two of our best performing antisense oligonucleotides (**14** and **15**) against motif 3 and compared them with two related mismatch-containing control oligonucleotides (**35** and **36**). It can be appreciated from Figure 4 that oligonucleotides **14** and **15** present more potent antisense activity than the corresponding

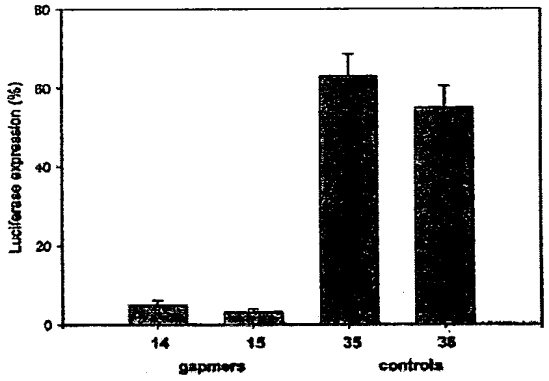

Figure 4. Down-regulation of Luciferase expression by oligonucleotides 14 and 15, and the corresponding mismatch-containing control oligonucleotides 35 and 36 directed against motif 3 at 2 nM.

mismatch-containing oligonucleotides **35** and **36**. In particular, we see a 14–21-fold loss in down-regulation for the mismatch-containing controls, thus being supportive for a sequence-specific mode of action.

Nuclease stability of α-L-LNA and β-D-LNA

The protection of oligonucleotides with LNA against nucleases has been shown (12,18). However, no systematic study of end-protection with α-L-LNA had been presented, which is a relevant issue in the design of oligonucleotides containing α-L-LNA. Therefore, we prepared oligonucleotides with α-L-LNA at the 3′-end ($t_{10}T^{\alpha}t$ and $t_9T^{\alpha}{}_2t$) and evaluated

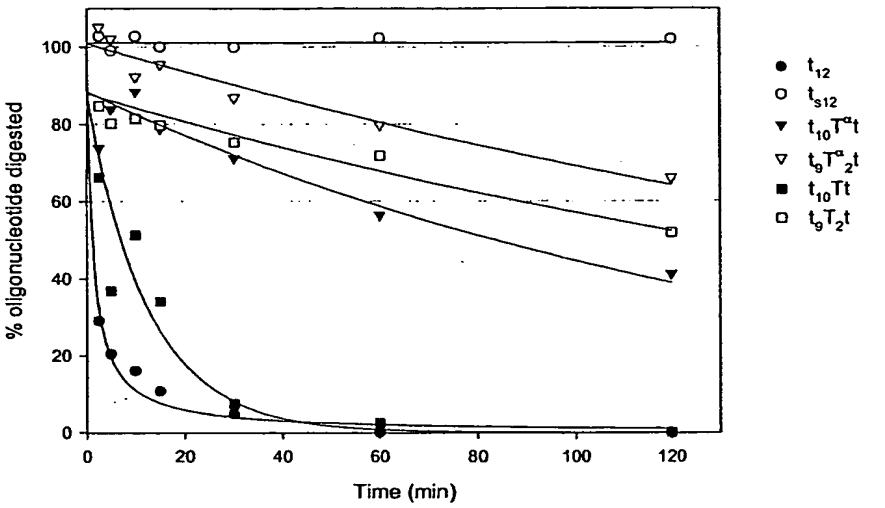

Figure 5. Kinetic study of oligonucleotides containing α-L-LNA at the 3′-end ($t_{10}T^{\alpha}t$ and $t_9T^{\alpha}{}_2t$) against a 3′-exonuclease (SVPD), in order to evaluate the nucleolytic stability. The graph also shows the corresponding controls (t_{12}, t_{s12}, $t_{10}Tt$ and t_9T_2t). The assays were performed using 26 μg/ml oligonucleotide, 0.3 μg/ml enzyme at 37°C in a buffer of 50 mM Tris–HCl, 10 mM $MgCl_2$, pH 8. Aliquots of the enzymatic digestion were removed at the indicated times. In a separate assay, the enzyme was shown to maintain its activity under these conditions for at least 2 h (data not shown). The oligonucleotides were synthesized on deoxynucleoside-support, t. Upper case for LNA and lower case for DNA.

their nucleolytic stability against a 3′-exonuclease (Snake Venom Phosphodiesterase, SVPD), and thus compared them with the corresponding β-D-LNA.

As previously noted (22), the introduction of just one β-D-LNA ($t_{10}Tt$), gives no increase in stability compared to DNA (t_{12}); however, the introduction of one α-L-LNA ($t_{10}T^{\alpha}t$) at the 3′-end of the oligonucleotide represents a significant gain in stability (40% full length oligonucleotide present after 2 h digestion) (Fig. 5). The introduction of two modifications ($t_9T^{\alpha}{}_2t$) contributes even more to the stability of the oligonucleotide (65% full length after 2 h digestion) and is superior to the corresponding β-D-LNA (t_9T_2t).

The gapmer is the most used design in antisense strategy with 2′-*O* modified nucleotides. This however has the disadvantage of turning the oligonucleotides more sensitive against endonucleases. Therefore, we wanted to evaluate the effect of an increasing deoxynucleotide gap on the stability against S1-endonuclease using 16mers with LNA containing flanks (Fig. 6).

We saw that fully modified oligonucleotides with β-D-LNA or α-L-LNA are very stable against S1-endonuclease. After 2 h digestion, most of the β-D-LNA (T_{16}) (85%) and α-L-LNA ($T^{\alpha}{}_{15}T$) (over 80%) remained, while the corresponding phosphorothioate could not be detected after 30 min digestion (Fig. 6).

Increasing the gap size results in a decrease in stability against S1-endonuclease (Fig. 6). As expected, no fragmentation of the LNA flanks was identified. It is important to note that with a gap of three DNAs, over 60% of the oligonucleotide is still full length after 120 min. This is significantly better than phosphorothioates, which in this assay are completely degraded in approximately 40 min.

This study was carried out with gapmers containing β-D-LNA in the flanks, but investigations with gapmers and chimeras (*vide infra*) containing α-L-LNA are currently under way.

Gapmers containing α-L-LNA

Bearing in mind that α-L-LNA affords more RNA:DNA like duplexes than β-D-LNA, we investigated the properties of oligonucleotides containing α-L-LNA both in the Luciferase assay and in the RNase H assay.

We started with a conservative approach, preparing different gapmers containing α-L-LNA, and evaluating them for their ability to down-regulate the target mRNA in the Luciferase assay. First, we substituted two β-D-LNA residues for two α-L-LNAs in a 16 gapmer against motif 3 with a thiolated 7 nt gap (PS gap), and placed the α-L-LNA in the junctions (the two residues in each flank next to the DNA gap, 22), in order to evaluate oligonucleotides that contain a low number of α-L-LNA. Then, we fully substituted both flanks with α-L-LNA and evaluated the effect (23–25).

Oligonucleotide 22 shows potent antisense activity. It is actually 5-fold more potent than the corresponding all β-D-LNA gapmer (9, gap of 7 nt) (Fig. 7). Also, at 2 nM an increase in activity compared to the β-D-LNA reference is observed.

The second design (all α-L-LNA in both flanks, 23 and 25) presents slightly better down-regulation levels than observed for β-D-LNA gapmers, both at 2 and 50 nM. As observed for β-D-LNA gapmers, a higher load of PS interlinkages correlates with a better antisense activity (Fig. 7).

The introduction of α-L-LNA proves to be a potent tool for the construction of different gapmers, with good antisense activity. The placement of α-L-LNA in the junctions or in the flanks results in very potent oligonucleotides. Again all gapmers showed efficient RNase H recruitment (22 is shown in Fig. 8).

Further design possibilities with α-L-LNA

We have shown that oligonucleotides containing α-L-LNA in a gapmer design present potent antisense activity, both with

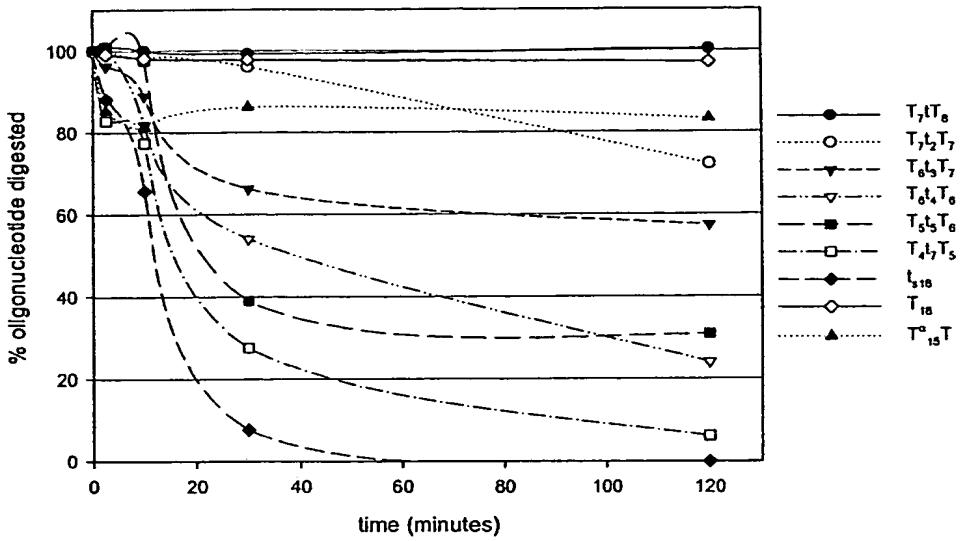

Figure 6. Kinetic study of different oligonucleotide gapmers (16mers) with an increasing deoxynucleotide gap against S1-endonuclease, in order to evaluate the nucleolytic stability. The graph also shows the stability of t_{s16}, T_{16} and $T^a{}_{15}T$ against S1-endonuclease. The assay was performed using 1.5 µM oligonucleotide and 16 U/ml enzyme at 37°C in a buffer of 30 mM NaOAc, 100 mM NaCl, 1 mM $ZnSO_4$, pH 4.6. Aliquots of the enzymatic digestion were removed at the indicated times. In a separate assay, the enzyme was shown to maintain its activity under these conditions for at least 2 h (data not shown). Upper case for LNA and lower case for DNA.

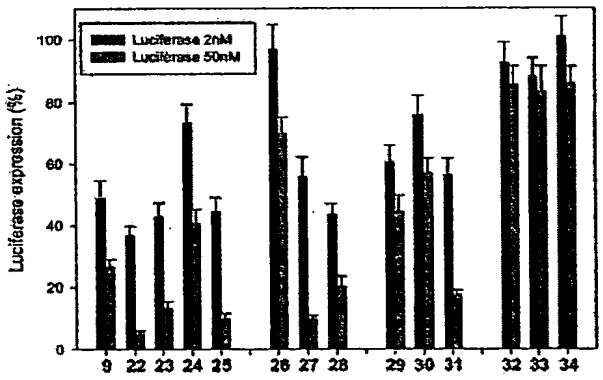

Figure 7. Down-regulation of Luciferase expression by different gapmers (**9**, **22**–**25**) and chimeras (**26**–**34**) containing α-L-LNA and β-D-LNA at 2 and 50 nM. Note: oligonucleotides with and without FAM were tested and compared, and no significant difference was appreciated between the free and FAM-labeled ones.

fully substitution of the flanks and with α-L-LNA only in the junctions of the flanks. Furthermore, since the ability of α-L-LNA for recruiting RNase H (18) was anticipated for different chimeras, we decided to further explore the possibilities. α-L-LNA in different chimeras (a chimera comprises an alternate composition of DNA, β-D-LNA and/or α-L-LNA) was tested for their antisense activity in the Luciferase assay and for RNase H recruitment, which will be described in the next section. The equivalent chimeras exclusively containing β-D-LNA were also prepared, in order to establish a comparison. Table 1 illustrates the chosen designs of chimeras against motif 3.

The first chimera consisted of a gapmer (with a 7 nt gap) containing β-D-LNA in the flanks and one β-D-LNA residue in the middle of the gap (**26** in Table 1). This turns the 7 nt gap into two 3nt gaps and thus should provide the oligonucleotide with further stability against nucleases. The presence of just one β-D-LNA residue interrupting the stretch of DNAs in the gap results in a dramatic loss of down-regulation (**26**, Fig. 7). We evaluated the antisense activity of the corresponding chimera, presenting an α-L-LNA residue interrupting the DNA stretch (**27**). Using α-L-LNA, the design shows significant down-regulation at 50 nM concentration of oligonucleotide, thus achieving significant down-regulation with a DNA gapsize of only 3 nt residues (Fig. 7). Chimera **28**, which contains two α-L-LNA residues at the edges of the flanks and one α-L-LNA residue interrupting the gap, also shows potent levels of down-regulation compared to **26**.

Next, we evaluated the interruption of the DNA stretch with two LNA residues, comparing the effect of using β-D-LNA or α-L-LNA. In **29**, we placed two β-D-LNA residues interrupting the gap. This resulted in poor antisense activity. No gain in antisense activity was recorded by placing two α-L-LNAs in the gap (**30**). However, a significant gain at 50 nM was obtained when both the two residues in the gap and all the flanks were substituted with α-L-LNA (**31**) (Fig. 7).

We also included highly substituted α-L-LNA oligonucleotides, in order to further explore the possibilities of α-L-LNA. Chimera **33** is based on a design containing 2′-deoxy-2′-fluoro-β-D-arabinonucleosides (2′-F-ANA) (23) for which Damha *et al.* claimed that the ability to elicit RNase H degradation of target RNA is significantly better than for the all DNA antisense oligonucleotides and for a 2′-F-ANA/DNA gapmer construct. Since α-L-LNA also shows partial RNase H

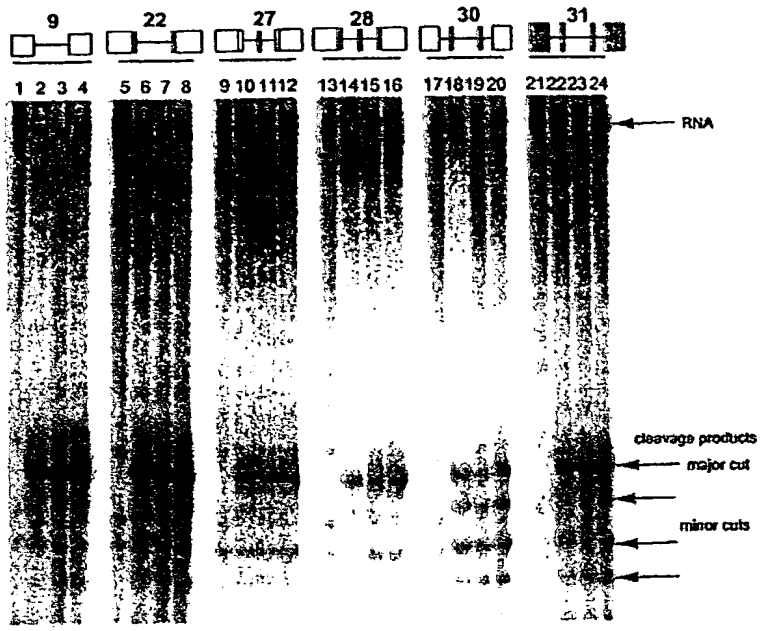

Figure 8. Electrophoresis analysis of ^{32}P-labeled target RNA degradation products. The chimeras (27, 28, 30, 31 and gapmer 22) containing α-L-LNA and the labeled RNA were incubated with *E.coli* RNase H1 and aliquots were taken at 0, 10, 20 and 30 min, electrophoresed and reaction products visualized by autoradiography. A gapmer (9) containing β-D-LNA was included in the gel for reference. In the drawing, the line is DNA, the rectangle LNA and the gray shadow corresponds to α-L-LNA residues.

recruitment, that design might also be a good design using α-L-LNA. Oligonucleotide **34** was also evaluated in terms of antisense activity. Interestingly, highly modified chimeras were inefficient in directing down-regulation (Fig. 7). Oligonucleotide **33** was also evaluated in a RNase H assay and showed no cleavage under our experimental conditions (data not shown). This most probably accounts for its poor antisense activity.

The affinity (T_m) of all oligonucleotides containing α-L-LNA was measured against complementary DNA. Even though α-L-LNA is known to confer lower thermal stability to the oligonucleotide than β-D-LNA, it was still very high for all of them (T_m > 60°C).

RNase H recruitment of the chimeras containing α-L-LNA

Since RNase H recruitment is a significant property of an antisense oligonucleotide, most oligonucleotides of this study were further evaluated in a RNase H assay, as already mentioned. It was especially interesting to evaluate the chimeras containing α-L-LNA. In Figure 8, we can see a representative electrophoresis gel for different chimeras containing α-L-LNA.

Lanes 1–4 (Fig. 8) show the very rapid degradation of the targeted RNA with a β-D-LNA gapmer (**9**, reference). Lanes 5–24 show the activation of RNase H with different chimeras (**27**, **28**, **30**, **31** and gapmer **22**) containing β-D-LNA and/or α-L-LNA in the flanks and α-L-LNA interrupting the gap. In all cases, RNase H activation is observed. However, preferences in cleavage sites varied depending on the design (minor cuts). Typically, they were at the residue complementary to the last DNA residue before the flank towards the 5′-end of the antisense oligonucleotide (major cut), and in the middle of the gap. Other minor cuts were found, always at the sites complementary to the DNA gap.

In our set-up, the RNase H assay has been shown to be not very discriminative, and has only been used as a first and non-restrictive filter in the selection of most potent antisense oligonucleotides. No prediction of antisense activity can be anticipated just from this assay.

CONCLUSIONS

From our study, we conclude that the optimal gap size for a gapmer (16mer) containing β-D-LNA lies between 7 and 10 nt. A further determination (discrimination between 7 and 10 nt) is motif-dependent and relates to a delicate balance between affinity and gap size. The high affinity caused by the introduction of β-D-LNA enables the use of potent antisense oligonucleotides as short as 12 and 14 nt in size. Compared to β-D-LNA, α-L-LNA shows superior stability against a 3′-exonuclease. α-L-LNA proved to be a potent tool enabling the construction of different gapmers and chimeras, which present potent antisense activity. The presence of α-L-LNA in the gap and in the junctions results in oligonucleotides with potent antisense activity, thus potentially conferring stability to the oligonucleotides against nucleases.

In conclusion, both β-D-LNA and α-L-LNA are shown to be excellent in the design of antisense oligonucleotides with potent antisense activity. Furthermore, α-L-LNA presents more versatile design possibilities.

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